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14. ABSTRACT

This report covers the second year of a three-year project that examines the hypothesis that the healing of critical size femoral defects in the rat in response to recombinant, human bone morphogenetic protein-2 (BMP-2) is enhanced by "reverse dynamization". In reverse dynamization, the defect is initially fixed loosely. Once healing has commenced, stiffness is increased. This is the opposite of current clinical practice of fixing osseous defects as stiffly as possible. When dynamization is used in the standard fashion, it starts with high stiffness fixation and is followed by low stiffness fixation.
During the first year of the study, we provided proof of principle data demonstrating that reverse dynamization indeed accelerated healing of a rat femoral defect in the presence of BMP-2, and improved bone quality. This covered most of Specific Aims 1 and 2 of the original proposal.

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INTRODUCTION

This report covers the second year of a three-year project that examines the hypothesis that the healing of critical size femoral defects in the rat in response to recombinant, human bone morphogenetic protein-2 (BMP-2) is enhanced by "reverse dynamization". In reverse dynamization, the defect is initially fixed loosely. Once healing has commenced, stiffness is increased. This is the opposite of current clinical practice of fixing osseous defects as stiffly as possible. When dynamization is used in the standard fashion, it starts with high stiffness fixation and is followed by low stiffness fixation.

During the first year of the study, we provided proof of principle data demonstrating that reverse dynamization indeed accelerated healing of a rat femoral defect in the presence of BMP-2, and improved bone quality. This covered most of Specific Aims 1 and 2 of the original proposal.

During the second year of the study, we have completed additional experiments necessary to prepare the work for publication. This was successful, and the data were published in the November issue of the *Journal of Bone and Joint Surgeons (American)* (see appendix).

After this was accomplished, the rest of the year 2 work focused on addressing Specific Aim 4:

To determine whether the optimized method reduces the amount of rhBMP-2 needed to recover full mechanical strength by the healed bone.

Below we describe our progress in fulfilling this aim.

BODY

Before evaluating the effects of reverse dynamization on the BMP-2 requirement of healing, it was necessary to establish a dose-response curve for BMP-2 using standard (non-dynamizing) fixators.

Critical size (5mm) defects were created in the femora of groups of rats using methods we have described previously. Defects were treated with 11 μg (standard dose), 5.5 μg or 1.1 μg BMP-2 delivered on an absorbable collagen sponge in the same manner as the INFUSE® product used clinically to enhance bone healing. Control defects received only the absorbable collagen sponge. Defects were examined by weekly X-ray until euthanasia at 8 weeks. After euthanasia, defects were analyzed by μCT and histology.

Figure 1 shows the X-ray images. Defects treated with 11 μg BMP-2 showed clear evidence of intra-lesional mineralization by 3 weeks and were completely bridged by 5 weeks. A dose of 5.5 μg also produced mineralization and bridging in about the same time-frame, but it appeared to be less uniform. For instance, at 8 weeks there remained a discontinuity at the distal end of the defect. A dose of 1.1 μg produced a small amount of mineralization, and no bridging occurred.
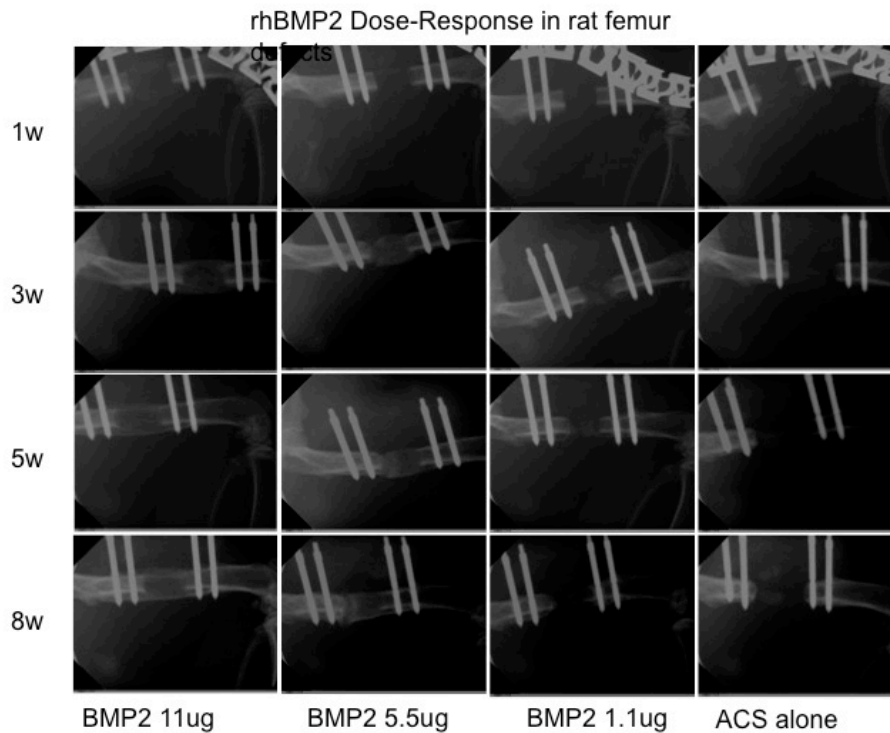
Figure 1. X-ray images of defects treated with different amounts of BMP-2.
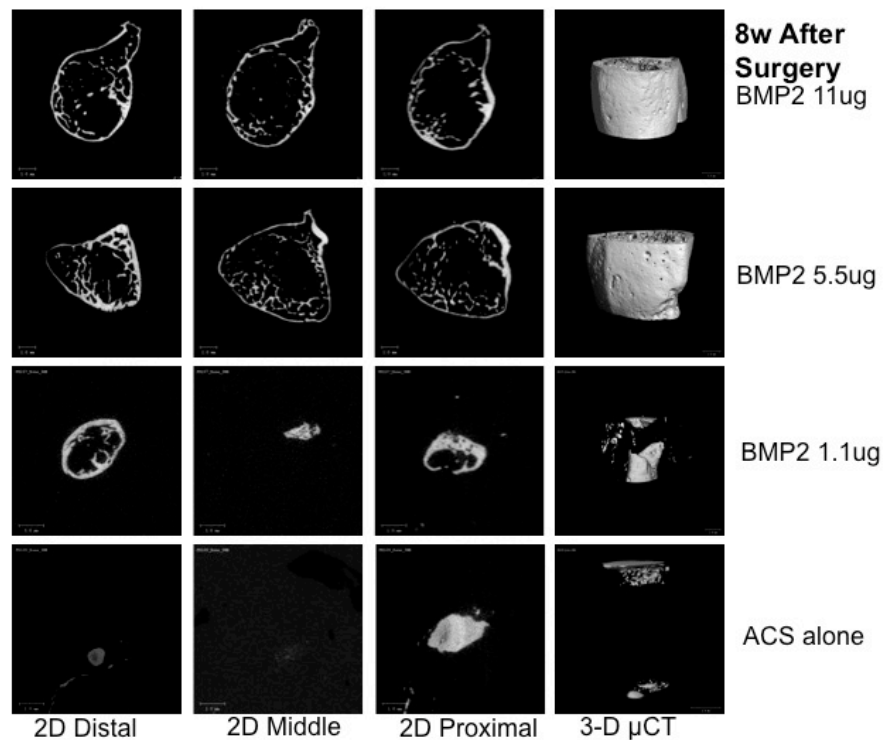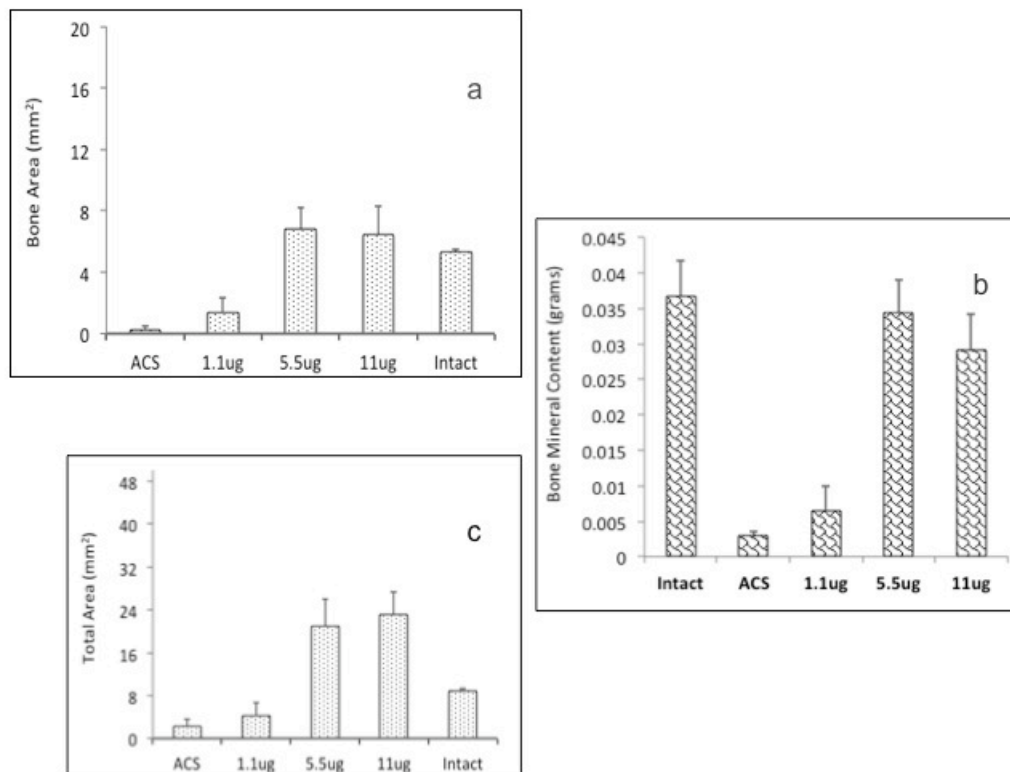ACS = Absorbable collagen sponge used to implant the BMP-2.

Figure 2. Micro-CT images of defects 8 weeks after treated with different amounts of BMP-2

The μCT data (figure 2) were consistent with the X-ray images. At 8 weeks, the 2 higher doses of BMP-2 resulted in the deposition of bone throughout the defect. The lowest dose of BMP-2, in contrast, caused the deposition of only small amounts of bone, which failed to fill the defect. Both the cross-sectional and 3-D images confirm that bone formed by 11μg BMP-2 is more uniform than bone formed in response to 5.5 μg BMP-2.

Quantitative analysis of the μCT data confirmed that the highest 2 doses of BMP-2 gave equivalent amounts of healing in terms of bone area (figure 3a), bone mineral content (figure 3b) and total area (figure 3c).

Figure 3.   Quantitative assessment of defects, 8 weeks after treatment with various doses of BMP-2.
a) Bone area   b) Bone mineral content   c) Total area.
Intact = Equivalent area on contralateral femur. ACS = Absorbable collagen sponge

Although the quantitative data did not differ between the highest 2 doses of BMP-2, histological examination (figure 4) confirmed the qualitative differences noted in the X-ray and μCT images. Staining of paraffin sections with hematoxylin and eosin confirmed that little bone was formed in the presence of 1.1 μg BMP-2, and none was present when only the absorbable collagen sponge was inserted. Although a large amount of bone was formed in the presence of 5.5 μg BMP-2, it was largely disorganized, woven bone with non-osseous soft tissue interspersed. The highest

dose (11 μg) of BMP-2, in contrast, drove the formation of new cortices containing marrow elements.
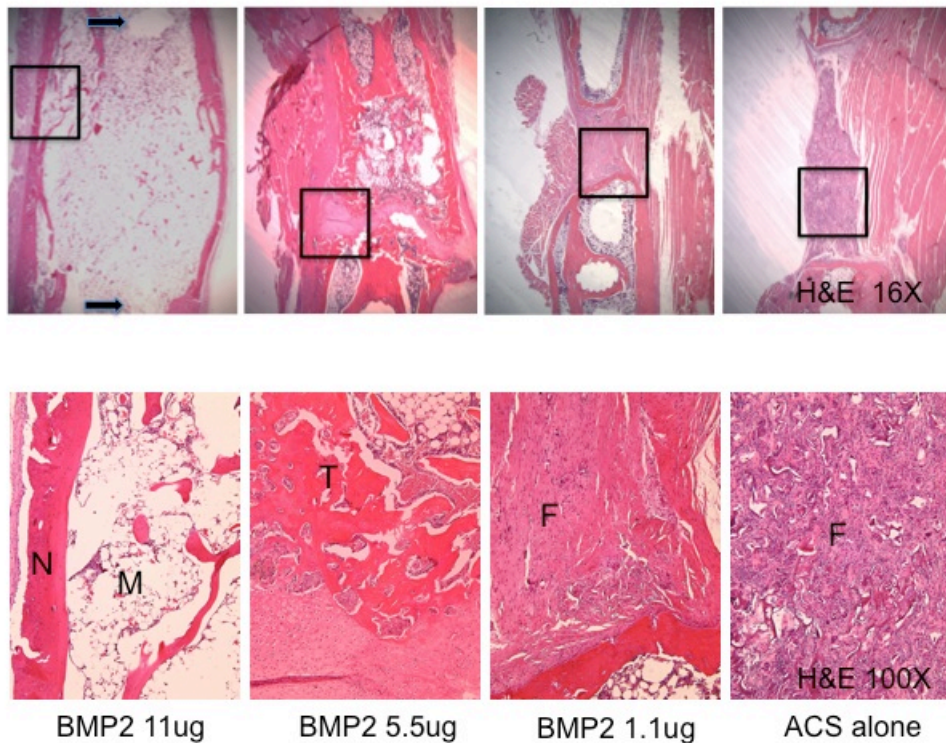
Figure 4. Histological appearance of defects at 8 weeks after implantation of various doses of BMP-2.
Top row: 16x magnification   Bottom row: 100x magnification
N= new cortex   M= marrow   T=trabecular bone   F= fibrous tissue
Boxed areas are shown in figure 5 with safranin orange-fast green staining.

When sections were stained with safranin orange-fast green to identify areas of cartilagenous tissue (figure 5) it was clear that substantial areas of cartilage remained in the defects treated with 5.5 μg BMP-2. These may well have accounted for the discontinuities noted in the X-ray and μCT images. No residual cartilage was observed in the defects treated with 11 μg BMP-2 or 1.1 μg BMP-2.

Because a dose of 5.5 μg BMP-2 showed an intermediate, sub-optimal ability to drive healing of the segmental defects, we decided to use this amount to study the effects of reverse dynamization. As shown in figure 6, X-ray examination revealed that reverse dynamization produced more uniform and complete healing than either low or high stiffness fixation. Histological examination with hematoxylin-eosin staining confirmed the greater maturity of the bone formed under reverse dynamization (figure 6). Staining with safranin orange-fast green showed that the amount of residual cartilage was much reduced under conditions of reverse dynamization (figure 7).
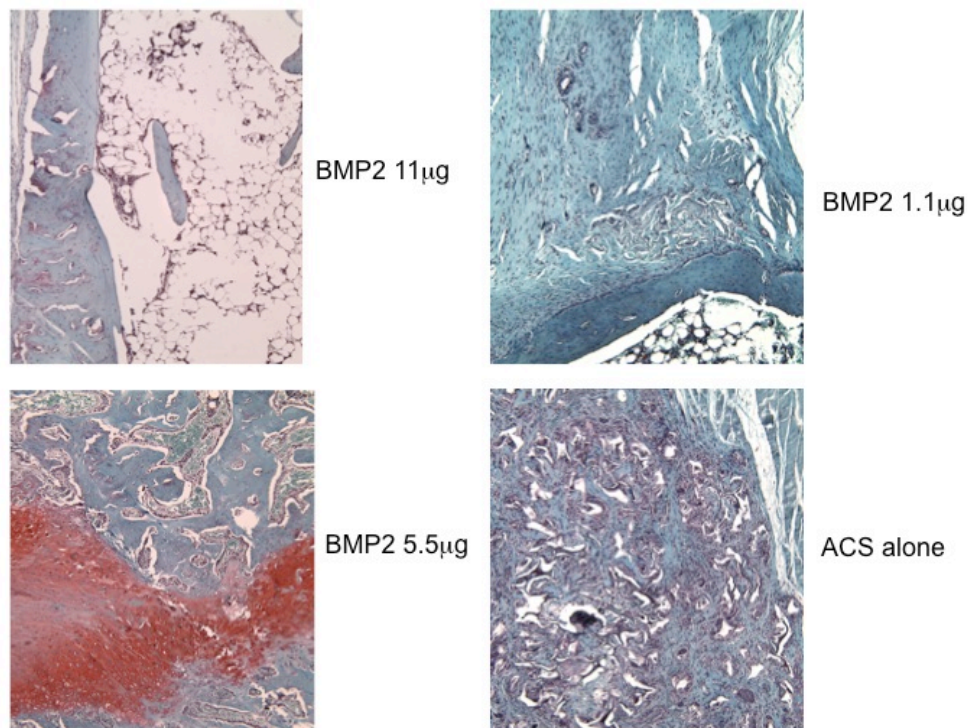
Figure 5. Histological appearance of sections stained with safranin orange-fast green 8 weeks after implantation of various doses of BMP-2
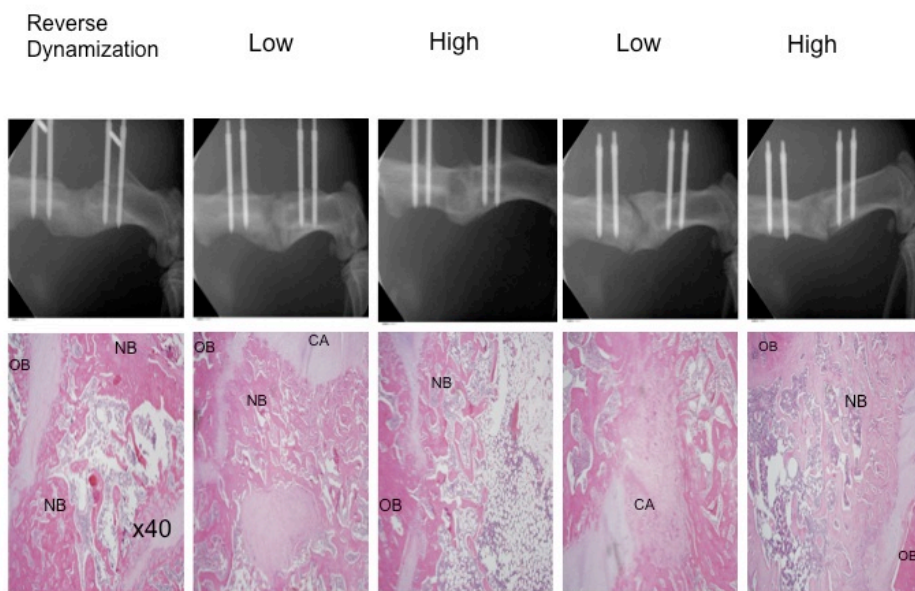
Figure 6. Effect of reverse dynamization on the healing of defects receiving 5.5 µg BMP-2
Top row: X-rays at 8 weeks of defects exposed to low stiffness (duplicates), high stiffness (duplicates) and reverse dynamization fixation.

Bottom row: Sections stained with hematoxylin and eosin.
NB= New bone   OB=old bone   CA=cartilage
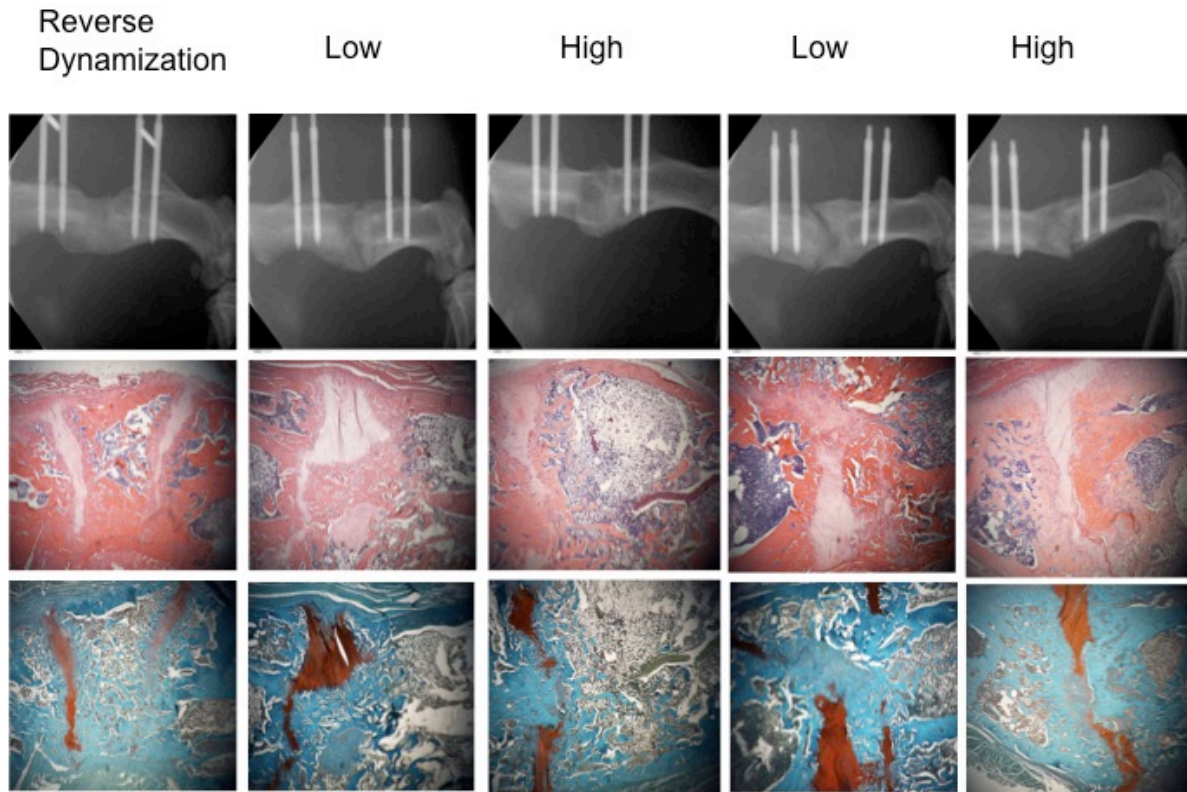
Figure 7. Higher magnification images of sections of defects healed with 5.5 µg BMP-2.
Top row: X-rays at 8 weeks of defects exposed to low stiffness, high stiffness and reverse dynamization fixation.
Middle row: Sections stained with hematoxylin and eosin.
Bottom row: Sections stained with safranin orange and fast green

KEY RESEARCH ACCOMPLISHMENTS
1. We have completed studies that confirm the superiority of reverse dynamization as a means of accelerating the healing of critical size defects in the rat femur.
2. We have completed the first series of experiments consistent with the hypothesis that reverse dynamization permits a lower dose of BMP-2 to be used to achieve healing.

REPORTABLE OUTCOMES
1. A poster reporting these data was presented at the 2012 Military Health System Research Symposium, 13-16 August in Fort Lauderdale, Florida.
2. A full-length paper was published in the *Journal of Bone and Joint Surgery.*

CONCLUSIONS
We confirm the efficacy of reverse dynamization as a means of accelerating bone healing and making BMP-2 more effective. Because it relies on an external fixator of a type already used in human clinical orthopaedics, this should be capable of rapid translation.

REFERENCES

Glatt V, Miller M, Ivkovic A, Liu F, Parry N, Griffin D, Vrahas M, Evans C: Improved healing of large segmental defects in the rat femur by reverse dynamization in the presence of bone morphogenetic protein-2. *J Bone Jt Surg Am* 94: 2063-2073, 2012

APPENDICES
Reprint of Glatt et al, 2012.

Improved Healing of Large Segmental Defects in the Rat Femur by Reverse Dynamization in the Presence of Bone Morphogenetic Protein-2

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Damian Griffin, MD, Mark Vrahas, MD, and Christopher Evans, PhD

Investigation performed at the Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Boston, Massachusetts

Background: Large segmental defects in bone do not heal well and present clinical challenges. This study investigated modulation of the mechanical environment as a means of improving bone healing in the presence of bone morphogenetic protein (BMP)-2. Although the influence of mechanical forces on the healing of fractures is well established, no previous studies, to our knowledge, have described their influence on the healing of large segmental defects. We hypothesized that bone-healing would be improved by initial, low-stiffness fixation of the defect, followed by high-stiffness fixation during the healing process. We call this reverse dynamization.

Methods: A rat model of a critical-sized femoral defect was used. External fixators were constructed to provide different degrees of stiffness and, importantly, the ability to change stiffness during the healing process in vivo. Healing of the critical-sized defects was initiated by the implantation of 11 μg of recombinant human BMP (rhBMP)-2 on a collagen sponge. Groups of rats receiving BMP-2 were allowed to heal with low, medium, and high-stiffness fixators, as well as under conditions of reverse dynamization, in which the stiffness was changed from low to high at two weeks. Healing was assessed at eight weeks with use of radiographs, histological analysis, microcomputed tomography, dual x-ray absorptiometry, and mechanical testing.

Results: Under constant stiffness, the low-stiffness fixator produced the best healing after eight weeks. However, reverse dynamization provided considerable improvement, resulting in a marked acceleration of the healing process by all of the criteria of this study. The histological data suggest that this was the result of intramembranous, rather than endochondral, ossification.

Conclusions: Reverse dynamization accelerated healing in the presence of BMP-2 in the rat femur and is worthy of further investigation as a means of improving the healing of large segmental bone defects.

Clinical Relevance: These data provide the basis of a novel, simple, and inexpensive way to improve the healing of critical-sized defects in long bones. Reverse dynamization may also be applicable to other circumstances in which bone-healing is problematic.

Large segmental defects of bone do not heal well and remain a clinical problem. Approaches to treating these defects include the use of autograft and allograft bone[1], distraction osteogenesis[2], and vascularized bone grafts[3], as well as the application of growth factors such as bone morphogenetic protein (BMP)-2 and 7, which are the active ingredients of INFUSE (Medtronic) and OP-1 (osteogenic protein; Stryker), respectively[4]. There is also interest in using osteoprogenitor cells[5], induced membranes[6], and tissue engineering[7,8]. Gene therapy technologies for bone-healing are in preclinical development[9]. The present study addresses modulation of the ambient mechanical environment as a way of promoting the healing

of large segmental defects experimentally with use of a rat model of a critical-sized femoral defect in conjunction with recombinant human BMP (rhBMP)-2.

Bone is highly responsive to mechanical loading, and there are a substantial number of studies on the effects of different mechanical regimens on fracture-healing[10,11]. Pioneering studies by Kenwright, Goodship, Perren, Claes, and others[10-15] have identified interfragmentary motion as the most important, mechanically determined parameter of fracture-healing. For instance, small, controlled, cyclic axial compressive displacement (stable fixation) enhances healing through a bigger callus and earlier fracture-bridging. In contrast, high strain forces (inadequate stability) inhibit callus formation. The effects of shear or transverse micromotion remain to be defined with precision.

Because different stages of the healing process respond differently to their mechanical environment, there has been much interest in the concept of dynamization, according to which the stiffness of fixation is reduced at a certain point during the healing process. This increases the interfragmentary motion and has been postulated to lead to more rapid remodeling of the regenerating bone. Dynamization at one week enhances healing of a 2-mm tibial osteotomy in dogs[16] but not a 1-mm femoral osteotomy in rats[17]. Using the latter model, however, Claes et al.[18] showed that late dynamization at three and four weeks enhanced healing.

In contrast to the above examples, no previous publications, to our knowledge, have described the influence of the ambient mechanical environment on the healing of critical-sized segmental bone defects. We performed studies using a rat model of a critical-sized femoral defect. These defects do not heal spontaneously, but they heal in response to BMP-2. External fixators were designed to provide different stiffnesses, with the ability to change the stiffness during the healing process. rhBMP-2 was used to stimulate healing of the defects. The literature suggests that large segmental defects in the rat heal in response to BMP-2 by an endochondral process[19]. Because shear forces are known to promote chondrogenesis[20], we hypothesized that a low-stiffness fixator would promote the early formation of cartilage. We further hypothesized that a subsequent increase in fixator stiffness would provide the rigidity needed for the efficient ingrowth of blood vessels and other aspects of the endochondral process. Thus, we suggested that healing of a large segmental defect in response to BMP-2 would be improved by early loose fixation followed by subsequent stiff fixation once bone begins to form. We term the transition from a less stiff fixation to stiffer fixation reverse dynamization.

Materials and Methods
Study Design
Pilot Study

External fixators of three different stiffnesses were constructed as described previously[21] and used in a pilot study to determine their influence on the first two weeks of bone-healing in the presence of BMP-2. This study had two aims: (1) to test our hypothesis that the fixator with the least stiffness would promote the most rapid early healing response and (2) to determine a suitable time for increasing fixator stiffness.

Thirty-six rats underwent surgery to create 5-mm femoral defects and were divided into three equal groups of animals that received low, medium, or high-stiffness fixators. All animals received BMP-2. Radiographs of the femoral defects were made after nine and fourteen days. Four rats per group were killed at six, nine, and fourteen days after surgery, and their femora were processed for histological analysis.

The subsequent reverse dynamization protocol was determined by the radiographic and histological data from the pilot study.

Reverse Dynamization

On the basis of the pilot study data, reverse dynamization was implemented by switching from low-stiffness to high-stiffness fixators at day 14. Healing of these animals was compared with that in animals whose low, medium, and high-stiffness fixators were not changed. A small number of control animals that did not receive BMP-2 were included to confirm that the defects did not heal spontaneously. Defects of all animals were monitored with weekly radiographs. At eight weeks, all animals were killed. All specimens were assessed with dual x-ray absorptiometry and microcomputed tomography (micro-CT). Nine
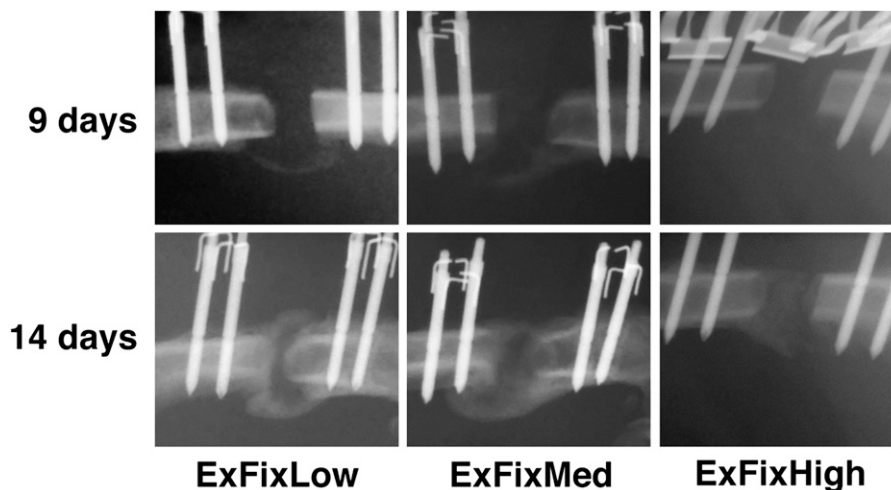

Fig. 1

Radiographs, made at nine and fourteen days, of defects stabilized with low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators.

specimens from each group were subjected to mechanical testing, and three were processed for histological analysis.

The design of this experiment is summarized in the Appendix.

Methods

Fixators

Defects were stabilized with custom-made external fixators, as described previously[21]. Their key features are interchangeable connection elements of different stiffnesses. The present work evaluated connection elements with stiffnesses of 114, 185, and 254 N/mm.

Surgery

A 5-mm, critical-sized, midfemoral defect was created in the right hind limb of each rat. We described this model previously, confirming that it does not spontaneously heal but heals when 11 μg of rhBMP-2 is inserted into the defect[22,23]. Groups of rats were maintained for eight weeks with each of these fixators. An additional group underwent reverse dynamization, whereby a low-stiffness fixator was applied for the first two weeks and then was switched to high-stiffness fixator (see Appendix). The number of control animals was low because of extensive historical data confirming that these defects do not heal

spontaneously[22,23]. The numbers of animals in the treatment groups were based on historical data confirming sufficient statistical power[22,23].

Animal care and experimental protocols were followed in accordance with National Institutes of Health guidelines and were approved by our institution's Institutional Animal Care and Use Committee.

The surgical procedure has been described in detail previously[21]. Briefly, male Sprague-Dawley rats weighing between 325 and 360 g were anesthetized with isoflurane (2% with 2 L/min O_2 by air mask). Before surgery, each rat was given an antibiotic (20 mg/kg of cefazolin) and the analgesic buprenorphine (0.08 mg/kg) intramuscularly in the left leg. An incision of approximately 3.5 to 4 cm was made through the skin, and the shaft of the femur was exposed.

The external fixator bar was used as a positioning guide to permit reproducible positioning of four drill-holes to accommodate the screws used to secure the fixator. After the fixator was in place, a saw guide was used to make the 5-mm segmental defect with use of a 0.22-mm wire Gigli saw. After the defect was created, the saw guide was removed and a collagen sponge impregnated with rhBMP-2 (11 μg in 100 μL of saline solution) was added to the defect area. Control defects received a sponge lacking BMP-2. The wound was closed in layers. On the first three postoperative days, the rat was given analgesic every twelve hours and antibiotic every twenty-four hours.
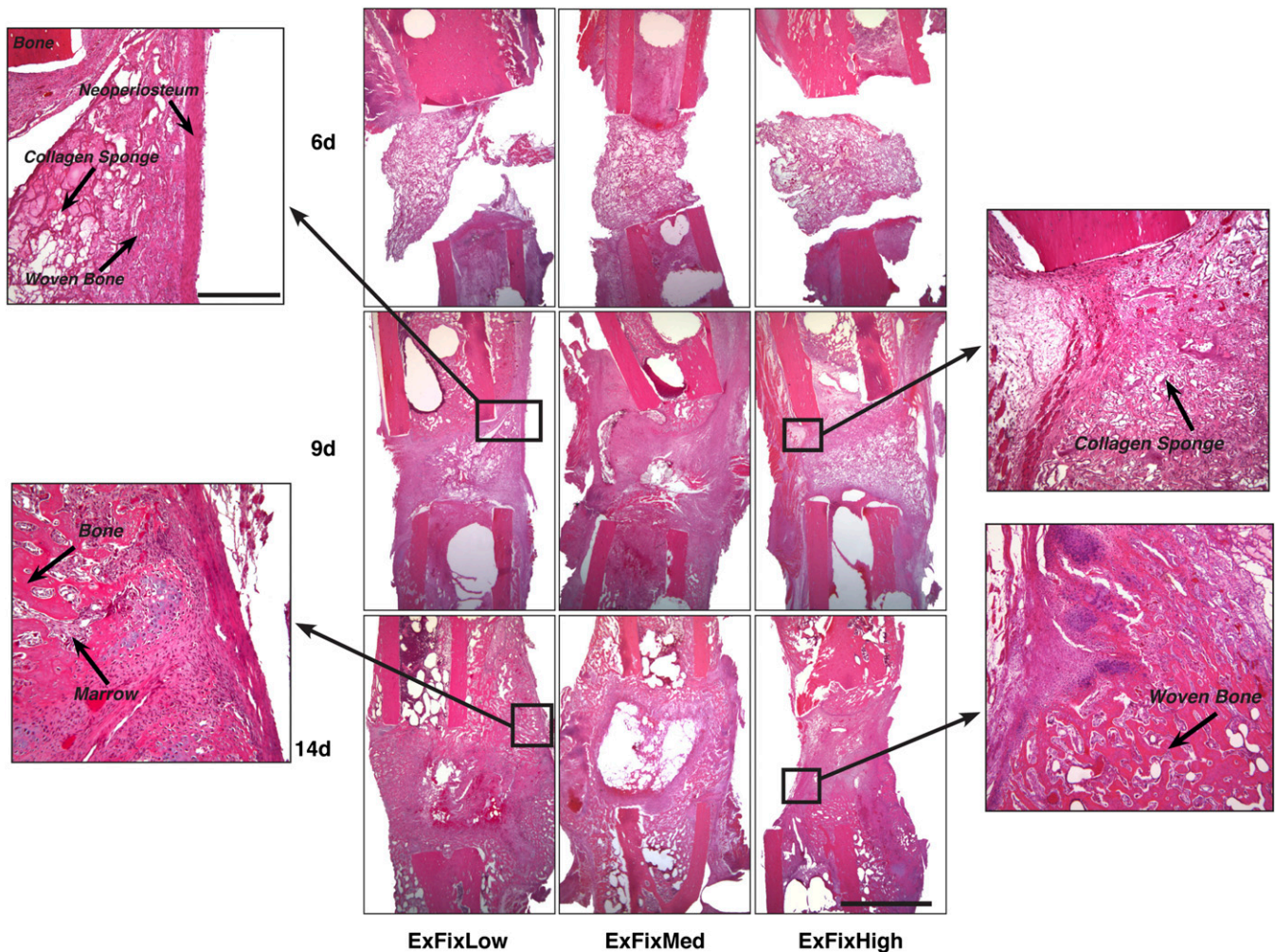

Fig. 2-A

Figs. 2-A and 2-B Histological appearance of defects stabilized with low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators at six, nine, and fourteen days. Bars indicate 2 mm in low-magnification images and 0.5 mm in high-magnification images. **Fig. 2-A** Hematoxylin-eosin staining.

Radiographic Evaluation

Bone-healing was evaluated with serial radiography with use of a digital dental x-ray unit (Heliodent DS; Sirona, Bensheim, Germany). While under general anesthesia, the rats were placed in a ventral position and the hind limb was laterally rotated so that the external fixator was not in the path of the x-ray source.

Histological Analysis

Femora were fixed for histological analysis in 4% ice-cold paraformaldehyde and were decalcified for six to eight hours in RDO Rapid Decalcifier (Apex Engineering, Aurora, Illinois), testing with a needle as the decalcification proceeded. Fixed and decalcified tissues were dehydrated in graded ethanol up to 100%, transferred to xylene, and embedded in paraffin. Five-micrometer paraffin sections were placed on poly-L-lysine-coated slides, dried overnight, and evaluated immediately or stored at 4°C. Sections were stained with hematoxylin-eosin or safranin orange-fast green.

Microcomputed Tomography

Femora were scanned with use of a desktop microtomographic imaging system (μCT40; Scanco Medical, Bassersdorf, Switzerland) equipped with a 10-mm focal spot microfocus x-ray tube. Femora were scanned with use of a 20-μm isotropic voxel size, at 55 keV of energy, 200-ms integration time, with approximately 720 micro-CT slices per specimen. Evaluation was done only in the 4-mm (200 slices) central defect region to ensure that no preexisting cortical bone was included in the analyses. To evaluate the region of interest, we assessed the following variables: total cross-sectional area or the callus size of the defect (TA in square millimeters), bone area (BA in square millimeters), and bone area over total area (BA/TA in square millimeters). Polar moment of inertia (in millimeters to the fourth power) was also calculated from micro-CT images. Images were thresholded with use of an adaptive-iterative algorithm, and morphometric variables were calculated from the binarized images using direct, three-dimensional techniques that do not rely on any prior assumptions about the underlying structure.

Dual X-Ray Absorptiometry

Bone mineral content (in grams) of the defect region was measured by dual x-ray absorptiometry (Lunar PIXImus II; GE Medical Systems, Madison, Wisconsin). Specimens were placed on a Lucite block during scanning to simulate soft tissue. The scans were acquired with use of the small-animal high-resolution mode.

Ex Vivo Torsion Testing

Specimens were tested to failure in torsion with use of a materials testing system (Synergie 200; MTS Systems, Eden Prairie, Minnesota) to determine the mechanical properties of the healed defect in shear. Before the test, both ends of each specimen were embedded in polymethylmethacrylate to provide a
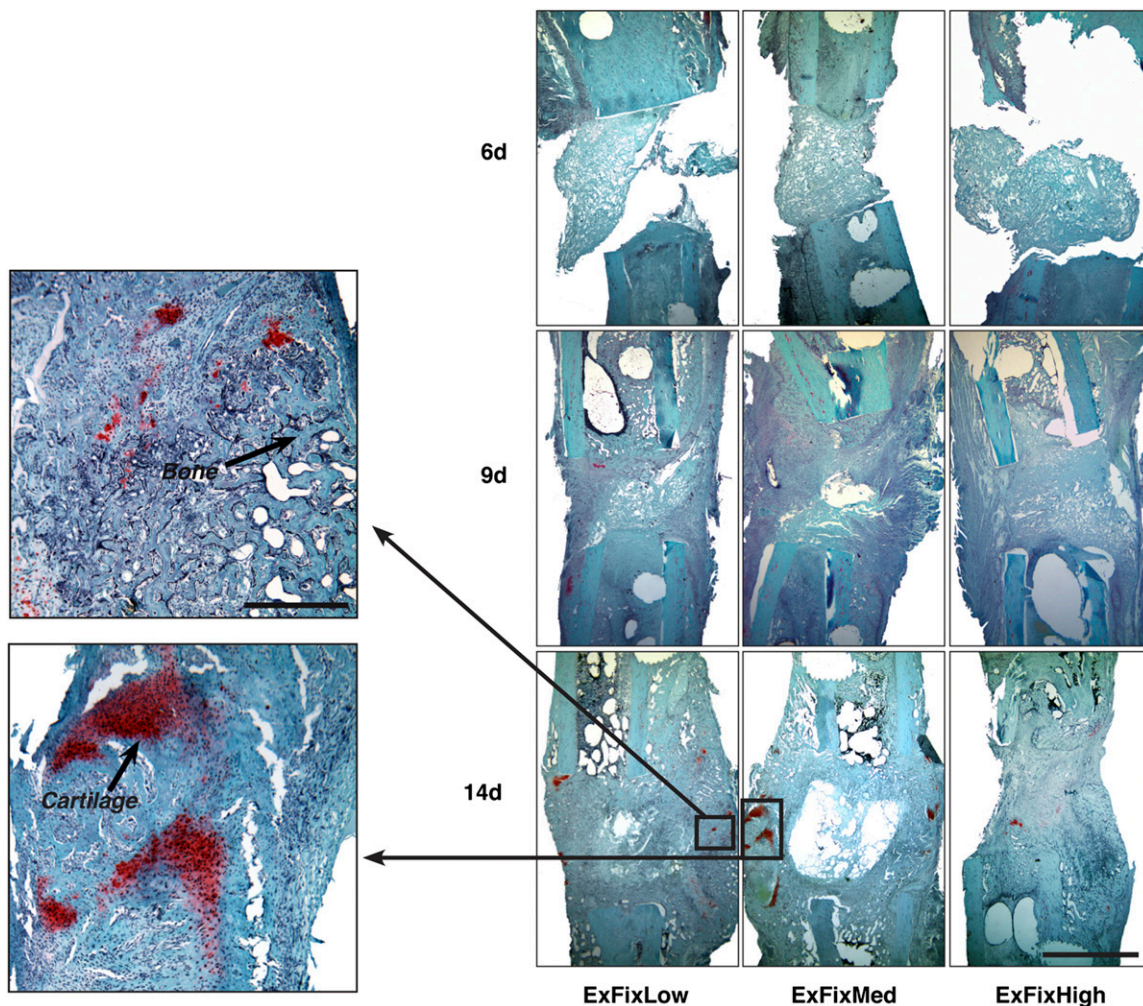

Fig. 2-B
Safranin orange-fast green staining.

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reproducible gripping interface with the testing fixture. All femora were tested to failure under regular deformation control and at the constant deformation rate of 5 rad/min. Angular deformation and applied load data were acquired at 10 Hz. The torque and rotation data were used to calculate the torsional stiffness and strength of the healed defect.

Sample Size and Statistical Analysis

The numbers of animals used were determined by our historical data using this model, in which eight to ten animals per group proved adequate[23]. A sample-size power analysis showed that the probability of detecting a significant difference with the numbers of animals per group used in our experiments was 95% for all of the imaging (twelve animals) and 85% for mechanical testing data (nine animals). The sample-size study detected a relationship between the independent and dependent variables at a two-sided 5% significance level. The power test confirmed that the animal numbers selected for each modality would be more than sufficient to detect significant differences.

Comparisons of continuous variables between two treatment groups were performed with use of a two-tailed t test, and comparisons between three groups were done with use of one-way analysis of variance. If the difference between the contralateral femur and the treatment groups was significant, a post hoc (Tukey) test was performed. A power analysis after the study was calculated to determine if we had sufficient animals per group for a significant difference. The power levels for all of the data were found to be from 0.8 to 1. Thus, the numbers of animals per group used in these studies is enough to determine a 5% difference between the test groups. All tests were two-tailed, with differences considered significant at $p < 0.05$. Data are presented as the mean and the standard error of the mean, unless otherwise noted.

Source of Funding

Results

Pilot Study

To determine a promising time for changing the degree of stiffness and to determine which stiffness fixators to use, we conducted a pilot experiment that focused on the early
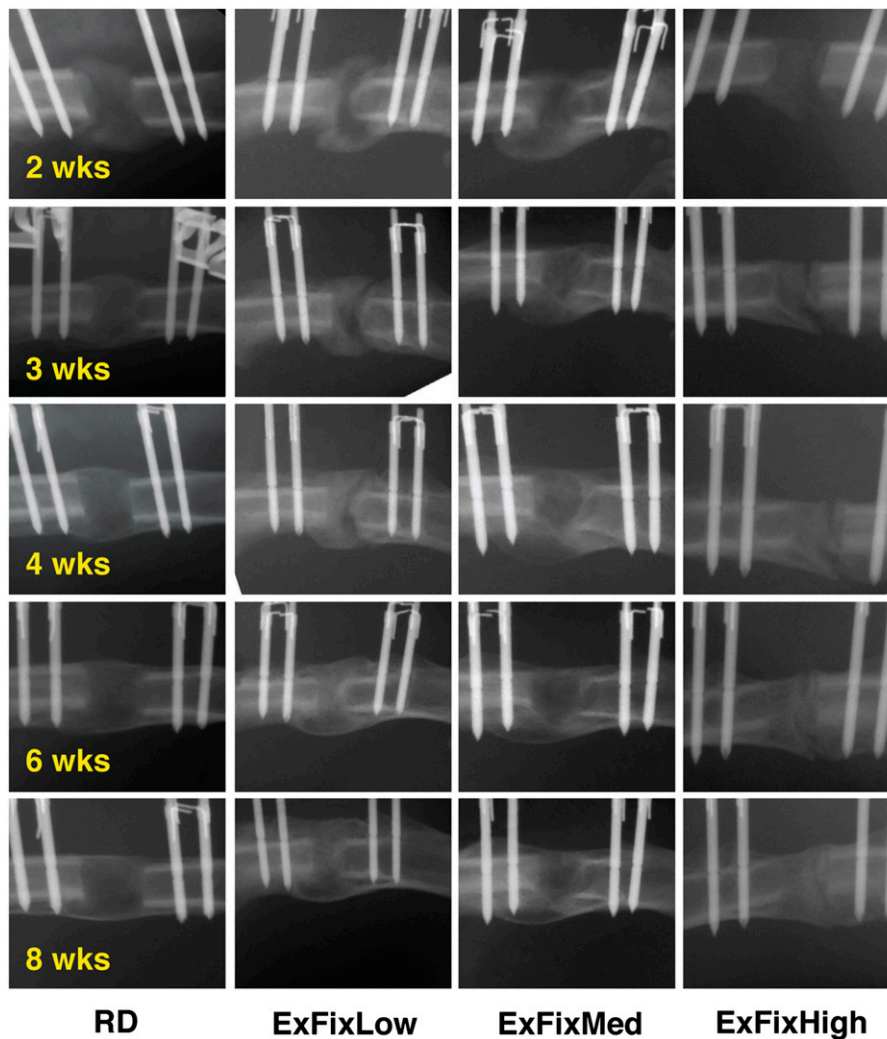

Fig. 3

Serial radiographs of defects stabilized with low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators and subjected to reverse dynamization (RD).

events in healing. There were three groups representing rats with segmental defects stabilized by low, medium, or high-stiffness fixators. All experimental defects received BMP-2.

Examination of the radiographic images at day 9 (Fig. 1) shows a faint crescent of radiopacity for the low-stiffness fixator group and, to a lesser degree, the medium-stiffness fixator group in the region opposite to the fixator. Little or no defined radiodensity was evident with the high-stiffness fixator group. At two weeks, this ranking was maintained with diffuse radiopacity in defects stabilized with the high-stiffness fixator and obvious, bridging radiopacity with the low-stiffness and medium-stiffness fixator groups. A visual, qualitative assessment of the radiographs suggested that the size of the radiodense callus was greatest for the low-stiffness fixator group and least for the high-stiffness fixator group.

Qualitative histological examination of the defects during this period was consistent with the radiographic findings (Figs. 2-A and 2-B). Staining with hematoxylin and eosin at six days suggested very little intralesional biological activity, and only the collagen sponge was visible in the defect. In the groups with the two lower-stiffness fixators, there was evidence of a periosteal reaction adjacent to the defect gap around the periosteum.

However, by nine days, the defects were filled with new tissue. In the low-stiffness and high-stiffness fixator groups, there was marked thickening of the periosteum, which appeared to migrate across the defect forming a bridge of neoperiosteum. This was more prominent on the side opposite to the fixator. With the low-stiffness fixator, and to a lesser extent the medium-stiffness fixator, there was evidence of new bone formation, often around the collagen sponge, with periosteal new bone formation on the bone adjacent to the defect. This presented as the formation of external callus, with the defect gap completely filled with soft tissue. Defects supported by high-stiffness fixators, in contrast, had no external callus and contained only fibrous soft tissue with little evidence of bone. Although, as in the other groups, there was marked woven bone formation along the periosteum adjacent to the defect, bridging did not occur.

At two weeks, qualitative examination of the histological sections suggested there was robust formation of woven bone with the low-stiffness fixator. Defects supported by the high-stiffness fixator appeared to contain only a little bone.

The defects stabilized with the medium-stiffness fixator had a distinctive gap in the middle of the defect. As described in the next section of the Results, the same feature was also observed on histological analysis and micro-CT images at eight weeks. Sections stained with safranin orange-fast green (Fig. 2-B) showed little evidence of cartilage formation, beyond a few isolated flecks, in defects stabilized with any fixator.

On the basis of these data, we decided, for the reverse dynamization stage of this study, to initiate fixation with the
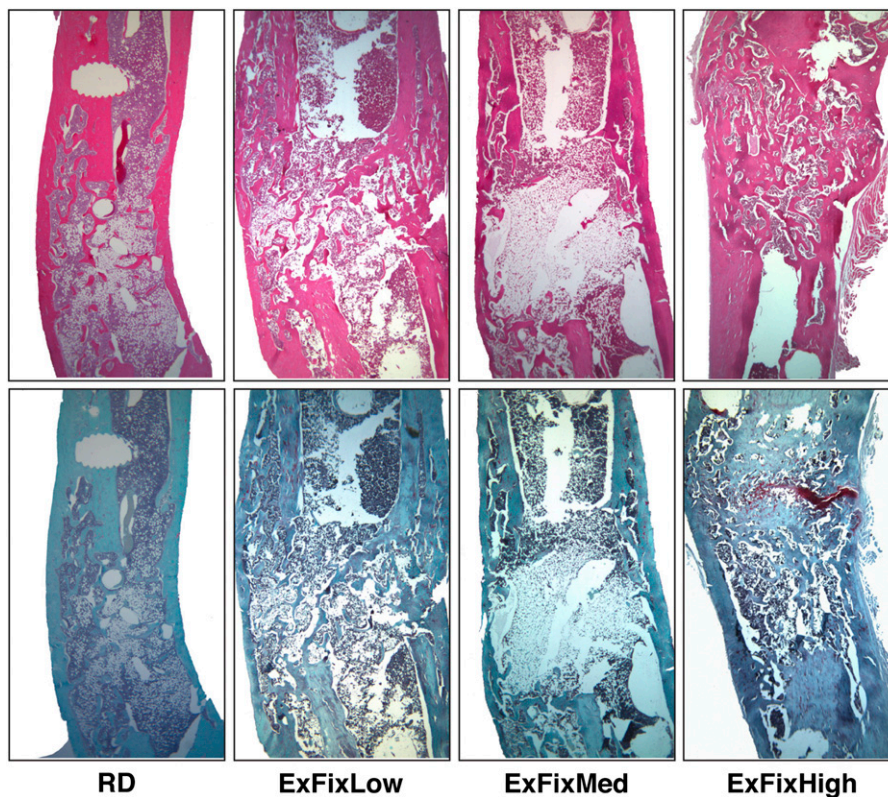

Fig. 4

Histological appearance of defects at eight weeks after stabilization with low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators and subjected to reverse dynamization (RD). The top row has hematoxylin and eosin staining, and the bottom row has safranin orange-fast green staining.

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low-stiffness fixator because it gave the most rapid early healing and to switch to the high-stiffness fixator at two weeks to promote the subsequent formation and remodeling of bone.

Reverse Dynamization

Control defects that did not receive BMP-2 (see Appendix) did not heal (data not shown). All other groups received BMP-2 (see Appendix) and mounted healing responses that varied

according to the stiffness of the fixator and whether it was dynamized. These responses are described below.

As noted above, two weeks after surgery, the 5-mm defect stabilized with the low-stiffness fixator contained considerable calcified tissue as shown on the radiographs (Figs. 1 and 3). At this point, fixator stiffness was changed from the low-stiffness to the high-stiffness fixator. One week later, the radiographs revealed complete callus bridging with osseous tissue and no

Fig. 5-A

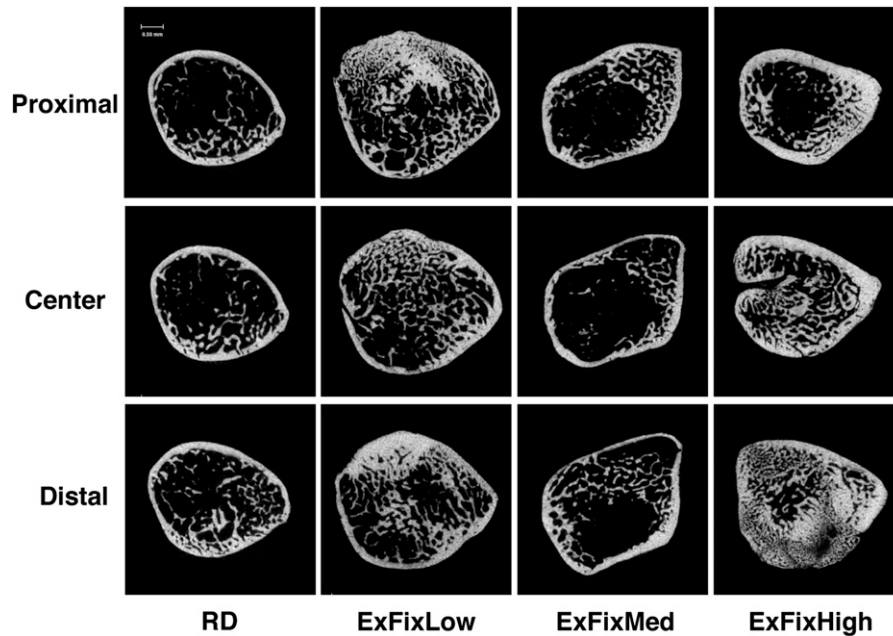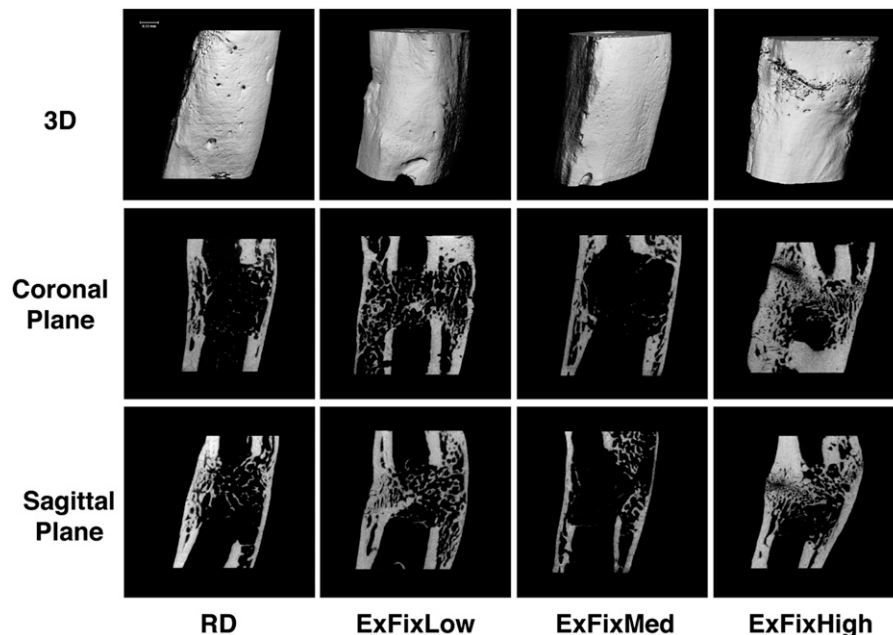Fig. 5-B

Figs. 5-A and 5-B Microcomputed tomographic images of defects at eight weeks after stabilization with low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators and subjected to reverse dynamization (RD). **Fig. 5-A** Cross-sectional images showing the proximal, center, and distal parts of the defect. **Fig. 5-B** Three-dimensional (3D), sagittal, and coronal images.
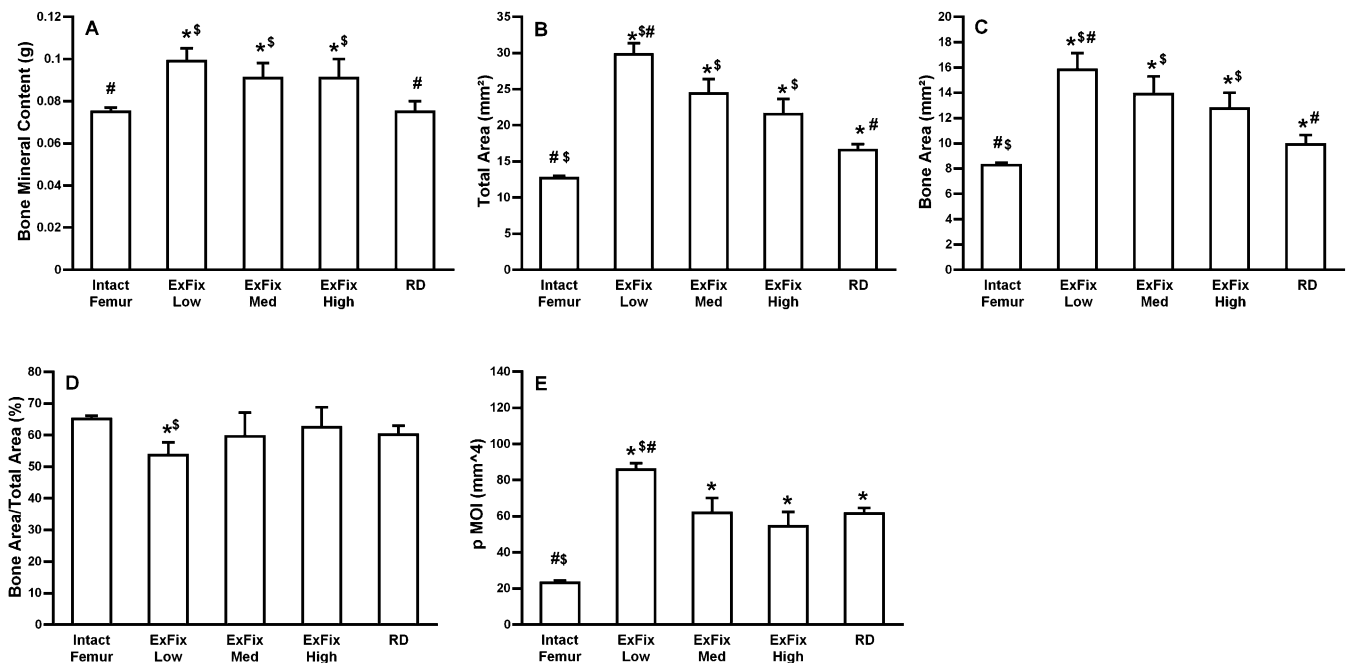
Fig. 6

Effect of low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators and reverse dynamization (RD) on dual x-ray absorptiometry and microcomputed tomographic (micro-CT) values at eight weeks. **Fig. 6-A** Bone mineral content (dual x-ray absorptiometry). **Fig. 6-B** Total callus area. **Fig. 6-C** Bone area (micro-CT). **Fig. 6-D** Bone area to total area. **Fig. 6-E** Polar moment of inertia (pMOI; micro-CT). Error bars indicate the standard error of the mean. #Significantly different from ExFixHigh. *Significantly different from intact, contralateral femur. $Significantly different from reverse dynamization group.

evidence of radiolucent lines in the reverse dynamization group. In contrast, soft tissue persisted until four weeks after the surgery in the defects stabilized with the low-stiffness and medium-stiffness fixators and for at least six weeks after surgery in the defects stabilized with the high-stiffness fixator.

After two weeks, the most obvious radiographic change in the reverse dynamization group was an apparent reduction in the width of the bone in the region of the defect by week 4. This phenomenon was not observed in the low-stiffness and medium-stiffness fixator groups until after six weeks. In the groups with the two lower-stiffness fixators, the width of the bone appeared to increase as healing progressed until the end of treatment, whereas in the group with the most rigid fixator, there was no change throughout the entire experiment.

The radiographs further suggest accelerated formation of new cortices in defects subjected to reverse dynamization. This is supported by the histological findings at eight weeks (Fig. 4). Defects subjected to reverse dynamization appeared to be narrower in cross section and had an organized tissue structure, with better architecture; well-formed, evenly distributed neocortices; and only limited trabecular bone, likely because of advanced remodeling. All other defects had persistent callus and contained disorganized woven bone with poor cortication. Defects stabilized for eight weeks with the medium-stiffness fixator maintained the central gap in the defect that was noted earlier (Figs. 2-A and 2-B) and was surrounded by unmineralized soft tissue. Defects stabilized for eight weeks with the high-stiffness fixator contained a prominent band of car-

tilage (Fig. 4), raising the possibility of development into a nonunion. Cartilage was not seen in any of the other groups at eight weeks.

The main conclusions histologically were confirmed by visual inspection of the high-resolution micro-CT images (Figs. 5-A, 5-B, and 6). The cross-sectional and longitudinal images shown in Figures 5-A and 5-B are consistent with the histological findings in showing more uniform and complete neocortices and less apparent trabecular bone, while appearing smaller in cross section. Reduced cross-sectional area was confirmed quantitatively (Fig. 6). These changes translated into images in which the cortical bone appeared thicker and trabecular bone less abundant compared with the low-stiffness, medium-stiffness, and high-stiffness fixator groups. Furthermore, in the reverse dynamization group, the new cortical bone had an even circumference over the entire length of the healed defect. This was not observed in the groups with constant stiffness.

Quantitative analysis (Fig. 6) confirmed that the bone mineral content of the reversed dynamization group was closer to normal values. The cross-sectional area of bone in the defects healed under reverse dynamization was only 19% higher than normal, whereas the cross-sectional areas in the other groups were considerably higher. The total cross-sectional area of the defects healed under reverse dynamization was also considerably lower. This is consistent with the smaller callus and higher degree of bone formation formed under reverse dynamization. The polar moment of inertia is a quantity used
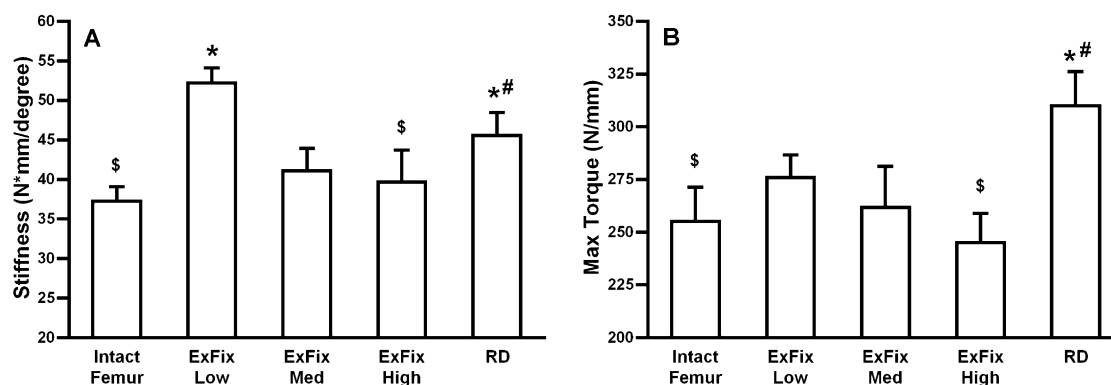
Fig. 7

Effect of low (ExFixLow), medium (ExFixMed), and high-stiffness (ExFixHigh) fixators and reverse dynamization (RD) on mechanical properties at eight weeks, showing data on stiffness (**Fig. 7-A**) and strength (**Fig. 7-B**). Error bars indicate the standard error of the mean. #Significantly different from ExFixHigh. *Significantly different from intact, contralateral femur. $Significantly different from reverse dynamization group.

to predict an object's ability to resist torsion. All experimental groups had polar moment of inertia values greater than those of the control femur.

The femoral defect stiffness following healing under reverse dynamization was significantly higher than that of the intact, contralateral femur and all experimental groups apart from the group stabilized with the low-stiffness fixator (Fig. 7-A). We attribute the high stiffness of the latter to the very large osseous callus (Fig. 4). Although this is disorganized, the sheer mass of bone endows considerable stiffness. Defects healed under conditions of reverse dynamization were considerably stronger than the intact femur and the defects stabilized with low-stiffness, medium-stiffness, and high-stiffness fixators (Fig. 7-B). The combination of high stiffness and unremarkable torque of the defects healed with the low-stiffness fixator means that they are more brittle than normal.

Discussion

These data support our hypothesis that reverse dynamization should improve the healing of a large segmental bone defect, but indicate a different mode of action from the one we suggested. In particular, initial stabilization of the defect with the least stiff of the tested fixators was predicted to promote chondrogenesis. Instead, little evidence of cartilage formation was seen during the first two weeks of healing. It is possible that the times selected for histological analysis (six, nine, and fourteen days) were not appropriate; however, in that case, endochondral bone formation would have to have occurred very rapidly between six and nine days after surgery.

Our erroneous assumption that healing of a large segmental defect in the rat femur in response to BMP-2 would occur via endochondral ossification stems from a report by Yasko et al.[19], who described this process in a similar rat model. Unlike our study, Yasko et al.[19] delivered BMP-2 on a demineralized bone matrix that could have supplied additional growth factors favoring chondrogenesis. Alternatively, mechanical factors may be responsible, given the high sensitivity of this system to the mechanical environment noted in the present work. Instead of using external fixation, Yasko et al.[19] employed a plate, which was likely quite stiff, although it was not mechanically characterized. In this regard, it is interesting that our stiffest fixator was the only one that led to substantial cartilage deposition.

Histological examination of the early period of healing provided additional valuable information. It was noteworthy that, six days after the insertion of a collagen sponge impregnated with BMP-2, the defects appeared biologically inert and only the sponge was visible. The only obvious biological response was activation of the periosteum adjacent to the cut ends of the defect. It is possible that the sponge prevented the formation of a hematoma and delayed the entry of osteoprogenitor cells. Three days later, the histological findings changed considerably, with defects filled with abundant soft tissue and evidence of osteogenesis in the groups in which low-stiffness and medium-stiffness fixators were used. This is intriguing, given that nearly all of the implanted BMP-2 would have disappeared within the first few days. Further research into the early response of segmental defects to BMP-2 is warranted to provide insight into the mechanism of healing.

The early differences in healing noted with the different fixators were still evident at eight weeks. The low-stiffness fixator produced the largest amount of bone and the high-stiffness fixator, the least. Little or no external callus was visible on radiographs at any time or by micro-CT or histological analysis at eight weeks, and the high-stiffness fixator, unlike the other fixators, generated substantial cartilage. This was evident as a cartilaginous band across the defect at eight weeks and raises concerns about an eventual nonunion. Since this cartilaginous tissue was not seen at the earlier time points (six to fourteen days), its formation appears to be related purely to the mechanical stimuli generated by the fixator and not by the BMP-2, which should have been absent. The medium-stiffness fixator produced the unusual effect of a gap in the center of the defect, which was visible histologically at early time points and was still present at eight weeks, where it could also be seen by micro-CT. This may be related to earlier observations that

healing with BMP-2 produces a "shell" of bone lacking internal osseous structure[24,25]. Of the fixators used continuously without dynamization during the eight-week experiment, the low-stiffness fixator provided consistently the best healing. This runs counter to the present clinical practice of fixing large segmental bone defects as rigidly as possible.

Reverse dynamization in combination with biological cues (e.g., BMP-2) offers numerous advantages in comparison with traditional bone-grafting techniques. There is no need for microvascular surgery, and donor-site morbidity with vascularized bone transfer and harvesting of cancellous autograft is obviated. Reverse dynamization avoids the pain, discomfort, and prolonged healing with distraction osteogenesis. The proposed method also potentiates the potency of a biological signal delivered to the site of the defect.

Reverse dynamization considerably accelerated maturation of the bone within the defect, which was evidenced on qualitative examination of the histological findings and micro-CT images as advanced formation of neocortices, reduced prominence of trabeculae, uniform contour, and accelerated reduction in apparent callus size. Consistent with these observations, quantitative data confirmed that the bone mineral content and bone area of the defects healed by reverse dynamization was closer to normal and had greater mechanical strength. Only one regimen of reverse dynamization was evaluated in this study, and it is possible that different stiffnesses or timing of reverse dynamization would provide even better results.

Although BMP-2 has shown preclinical efficacy in animal models, the clinical effectiveness of BMP-2 in healing of long bones has been disappointing. Our data suggest that an appropriate mechanical environment is necessary for BMP-2 to be effective, and research into the mechanobiology of cellular responses to BMP-2 could be fruitful. This, in turn, has three main components: mechanosensing, signal transduction, and effector cell response. These have been reviewed recently by Morgan et al.[26] in the context of fracture-healing.

Although these experiments used a rat model, the data are clear and unequivocal. The results are likely to be relevant to clinical orthopaedics, and it should be feasible to design and construct variable-stiffness fixators for use in humans. To our knowledge, we are the first to show that the healing of critical-sized segmental defects is highly responsive to the ambient mechanical environment and does not necessarily follow the same rules as fracture-healing in this regard. Further study of reverse dynamization could lead to improved clinical management of these difficult cases.

Appendix

(eA) A table showing the experimental design is available with the online version of this article as a data supplement at jbjs.org. ∎

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